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PRINCIPAL INVESTIGATOR: Dennis Rasmussen, PhD

CONTRACTING ORGANIZATION: Seattle Institute for Biomedical and Clinical Research
Seattle, WA 98108-1532

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Dennis Rasmussen, PhD

E-Mail: drasmuss@u.washinqton.edu

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SEATTLE INSTITUTE FOR BIOMEDICAL AND CU
1660 S COLUMBIAN WAY #151F
SEATTLE WA 98108-1532

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14. ABSTRACT

We have demonstrated that alcohol-na"ive rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. The development of increased alcohol intake and increased alcohol preference was highly correlated with acoustic startle amplitude previously determined before the initial access to alcohol. This study, which is key to this entire project, has been completed and is now in press. We have recruited, processed and trained a new staff person needed for the proposed studies and have also implemented new methods that were not in the original proposal but that make the studies more efficient and effective. One study that addresses administration of the a1-adrenergic receptor antagonist, prazosin, at the time of traumatic stress to prevent subsequent development of a rat PTSD-like syndrome has already been initiated in year 1 instead of the originally proposed start in year 3, due to the urgent need for new therapies to prevent PTSD in response to trauma. The remaining proposed studies using rat models to address stress and PTSD mechanisms as targets for pharmacotherapy of PTSD and alcohol abuse are proceeding on schedule .

15. SUBJECT TERMS

PTSD, alcohol, ethanol, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

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Table of Contents

1. INTRODUCTION: Studies from our research group demonstrating that the well-characterized, safe, well-tolerated and FDA-approved a1-adrenergic receptor antagonist (AR), prazosin, is effective not only in treating combat post-traumatic stress disorder (PTSD) symptoms but in decreasing alcohol drinking in both human and rat studies provide much-needed breakthroughs in the development of effective pharmacotherapies for alcohol use disorders as well as for PTSD. However, much work remains to determine conditions in which this treatment to reduce noradrenergic hyperactivation will be effective, characteristics of individuals who are most likely to respond, and underlying mechanisms providing bases for additional treatments. Our immediate objective is to identify key variables in rat models that will inform and complement human studies, providing a powerful translational approach for most efficiently and rapidly developing and implementing effective new pharmacotherapies for alcohol use disorders and co-morbid PTSD.

2. KEYWORDS: alcohol, ethanol, PTSD, prazosin, noradrenergic, startle, anxiety, stress, pharmacotherapy, prevention, rat, abuse

3. OVERALL PROJECT SUMMARY: There are no significant changes in the project goals or studies planned.
    - CURRENT OBJECTIVES There are 3 major goals, or Specific Aims, of this project. These objectives remain unchanged.
        - 1. Determine relationship of hyperexcitability, anxiety and a1-adrenergic receptor-mediated signaling to excessive voluntary alcohol drinking, *providing information from rat models that will likely reveal especially promising bases for:*

a) Prospectively identifying subsets of individuals who are highly vulnerable to developing alcohol use disorders (AUDs).

STATUS: We have demonstrated that alcohol-na"lve rats exhibiting high acoustic startle response (which is associated with increased anxiety-like behavior) develop increased subsequent alcohol intake and alcohol preference in an intermittent alcohol access (IAA) paradigm. This completed study, which is key to this entire project, has been published in the journal ALCOHOL AND ALCOHOLISM, and the paper is appended with this progress report. In this study, alcohol-na·ve male Wistar rats were characterized with an SR-LAB Acoustic Startle System to determine startle response to 10 presentations each of 40 ms 90, 95 or 100 dB white noise pulses at 30 s intervals, with one pulse of each intensity (i.e. 90, 95 or 100 dB in counterbalanced order within each 10 sequential sets of three pulses, with 60 dB background white noise between pulses. Three weeks after startle testing, 2-bottle choice access to 20% (v/v) alcohol vs water was provided for 24 h/day, 3 days/week (M, W, F) – i.e. and intermittent alcohol access (IAA) model. On days when alcohol was not provided, the rats had access to water only. After 36 alcohol access days (i.e. 12

weeks) alcohol intake and alcohol preference were determined over the next 3 alcohol access days to characterize the relationships of each rat's alcohol intake and alcohol preference relative to its pre-IAA acoustic startle responses. As illustrated in Fig. 1, startle amplitude in response to 95 or 100 dB stimuli was positively correlated with subsequent alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split) pre-IAA startle amplitude in response to 95 or 100 dB stimuli developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA, relative to rats with low pre-IAA startle amplitude.

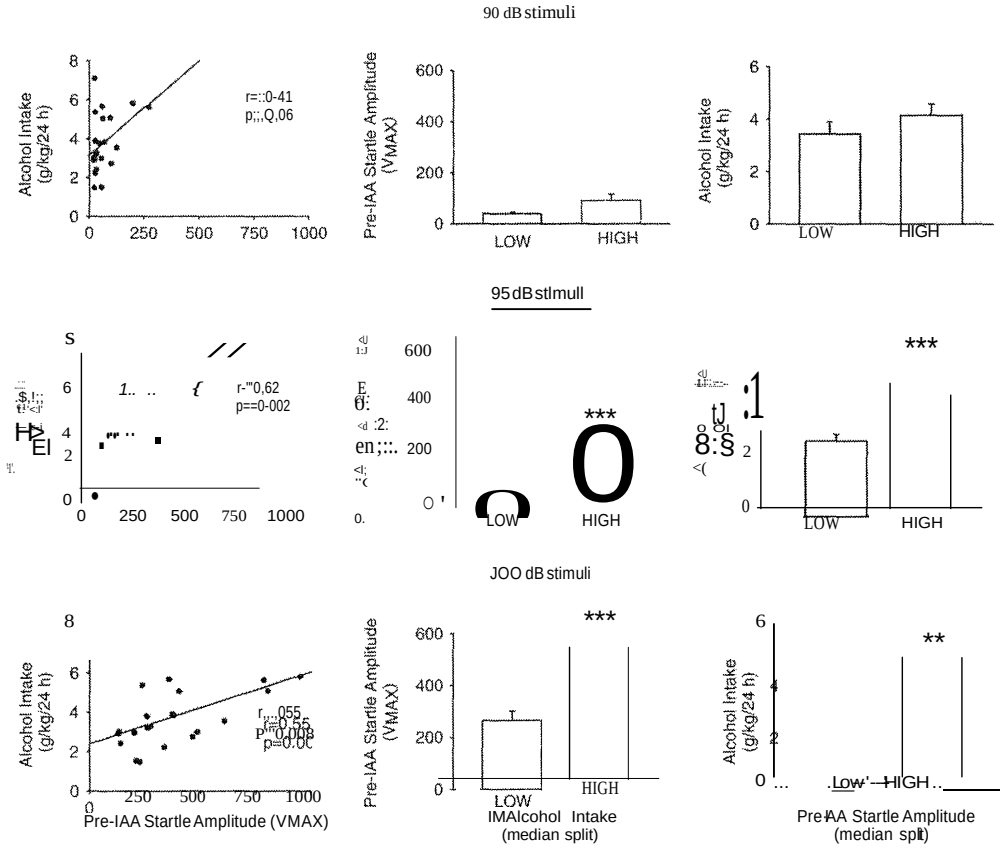

Fig. 1. Pre-IAA acoustic startle response vs alcohol intake following 3 months of IAA. Rows: The upper, middle and lower rows present analyses of pre-IAA responses to 90, 95 or 100 dB acoustic startle stimuli, respectively. Columns: The left panel in each row presents the correlation between pre-IAA acoustic startle amplitude vs IAA week 13 alcohol intake for all 22 rats. The center panel in each row presents the pre-IAA acoustic startle amplitude of rats grouped on the basis of low *vs* high (median split, n = 11 rats/group) alcohol intake in IAA week 13. The right panel in each row presents the IAA week 13 alcohol intake of rats grouped on the basis of low *vs* high pre-IAA acoustic startle amplitude.

**P :::; 0.01 *vs* Low, *** P :::; 0.001 *vs* Low. This work corresponds to Task 1, which was proposed to be completed by the end of year 1 and which is now complete.

b) predicting who is most likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work was not identified as a separate specific Task, but it is a component of several of the proposed experiments. Although our initial work suggests that high acoustic startle and associated increased anxiety-like behavior do predict increased suppression of alcohol drinking in response to prazosin, as hypothesized, we will investigate responses over all alcohol access and PTSD-like conditions of this overall investigation, as planned. Consequently, resolution of this issue will not be finalized until all experiments are completed. As described in the grant proposal, prazosin will be administered prior to voluntary alcohol drinking in rats that have been previously characterized for acoustic startle and anxiety-like behaviors in each of the experimental models used in these studies; it will be determined whether prazosin treatment disproportionately decreases alcohol drinking in those rats with pre-existing or PTSD-induced high acoustic startle and high anxiety-like behavior.

c) preventing initial acquisition of AUDs in prospectively-identified vulnerable individuals.

STATUS: This work corresponds to Task 2, which was predicted to be completed by the end of Year 1. It is hypothesized that rats exhibiting high acoustic startle before the initiation of IAA will subsequently exhibit high IAA alcohol intake (as we have demonstrated in Task 1), and that continuous treatment with prazosin before and throughout IAA will prevent this acquisition of high alcohol drinking. This experiment thus requires continuous treatment of the rats with prazosin during introduction of IAA. We had originally proposed to accomplish this with 3X/day oral treatments. However, more recent reports suggested that prazosin can be solubilized sufficiently to be administered with implantable osmotic minipumps to maintain prolonged (4 week) constant administration. We have now confirmed this in an unscheduled of the potential use of osmotic minipumps to administer the long-term prazosin treatments. This added work has confirmed that use of the minipumps with prazosin is feasible, providing more reliable and consistent drug administration and thus potentially improving results and facilitating extrapolation/translation to humans. Performance of this task (and Task 3, see below) has thus been improved, although final completion of the Task is behind the projected schedule (offset by the advance of Task 6, see below).

d) predicting who is most vulnerable to progression from voluntary to compulsive alcohol drinking. This work corresponds to Task 3, which was proposed to be completed at the end of year 2. The unscheduled characterization and implementation of the use of osmotic minipumps (discussed above) will also provide more reliable and consistent drug

administration for this study which requires prolonged administration of prazosin following prolonged IAA, with an hypothesis that prazosin will still suppress the high alcohol drinking in rats that had exhibited high acoustic startle and anxiety-like behavior before IAA and which had subsequently developed compulsive alcohol drinking (i.e., alcohol drinking that is maintained even after distasteful adulterants are added to the alcohol). This thus-improved experiment remains on schedule for year 2.

- 2. Evaluate PTSD/alcohol interactions, *providing information from rat models that will likely reveal especially promising bases for:*

a) determining cause-effect relationships between PTSD and AUDs, i.e., does PTSD increase vulnerability to developing AUDs and do AUDs increase vulnerability to developing PTSD?

STATUS: This work entails comparing production of a PTSD-like behavioral and acoustic startle profile in rats with *vs* without a previous recent history of alcohol liquid diet-induced excessive prolonged alcohol intake and dependence. This work corresponds to Task 4, projected to be conducted in year 2. The scheduling remains appropriate.

b) predicting who, among individuals with PTSD, is especially vulnerable to developing AUDs.

STATUS: This work will address whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent acquisition of increased IAA alcohol intake. This work corresponds to Task 5, projected to conducted in year 3. This scheduling remains appropriate.

c) predicting who, among individuals with PTSD, is likely to respond to prazosin with decreased alcohol drinking.

STATUS: This work addresses whether a rat PTSD-like behavioral and acoustic startle profile predicts subsequent effectiveness of prazosin in suppressing IAA alcohol intake. This work was not identified as a separate specific Task with a single proposed completion date, but it is a component of several of the proposed experiments, including Tasks 3, 4 and 5, which are not all projected to be completed until the end of year 3.

- 3. Determine whether the reduction of a1-AR mediated signaling at the time of traumatic stress will prevent the subsequent development of increased alcohol abuse and PTSD, informing whether prophylactic prazosin treatment is likely to decrease vulnerability to PTSD and alcohol use disorders.

STATUS: This work entails administration of prazosin at the time of traumatic stress to determine whether this treatment blocks subsequent development of a rat PTSD-like behavioral and acoustic startle profile, as well as associated increased subsequent IAA alcohol intake. This work corresponds to Task 6, which was projected to be conducted and completed in year 3. However, this Task has already been initiated and should be completed well ahead of schedule - due to urgency of developing effective preventive treatment to block development of PTSD.

- SUMMARY DISCUSSION: This first year was used for personnel recruitment and training, implementation of all necessary methodologies, completion of the first study (which is key to interpreting all subsequent studies), and initiating the subsequent studies - each of which is an individually long-term study (ranging from months to greater than a half-year for each of multiple temporally-overlapping cohorts of subjects within each study). This has gone well, and - although the start has been somewhat slower than predicted - the necessary groundwork for successful completion is done, and the overall project is on track for completing all goals.

4. KEY RESEARCH ACCOMPLISHMENTS
   - The key complete accomplishment in this first year is our demonstration that acoustic startle in alcohol-na"lve male rats predicts subsequent voluntary alcohol intake and alcohol preference, as detailed in the attached paper.

5. CONCLUSION: These results demonstrated in the completed key research accomplishment thus far are consistent with the hypothesis that is central to all other studies in this research project, i.e. that hyper-responsiveness characteristic of PTSD, alcohol withdrawal/abstinence, and increased noradrenergic activation contributes to - or at least is associated with - development of increased alcohol drinking, The results demonstrate that acoustic startle amplitude is an effective predictive index for subsequent increased voluntary alcohol intake and alcohol preference in the intermittent alcohol access model which is thought to be an effective model for investigating a variety of human alcohol use disorders. Acoustic startle response may be an especially useful index of the vulnerability to developing increased alcohol drinking because it is not dependent upon, and potentially confounded by, interactions with other behaviors. Importantly, acoustic startle is plso well-characterized for use in humans, providing translational utility. These results thus may provide a useful model for current and future investigations of neurobiological mechanisms mediating initiation and development of excessive drinking, as well as the mechanisms mediating co-morbidity of alcohol use disorders and PTSD - which is likewise characterized by increased startle response and behavioral hyperreactivity. Furthermore these results provide the conceptual basis for a potential approach to prospectively identifying individuals – including individuals with PTSD - at increased risk for future alcohol use disorders, thus allowing development and implementation of potential preventive intervention.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS
   a. Manuscripts

      Lay press: Nothing to report

Peer-Reviewed Scientific Journals: Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-na'lve male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065) [note: this paper was listed as *in press* in the original version of this progress report but has since been published, so I am now providing the citation for the published version].

Invited Articles: Nothing to report

Abstracts: Nothing to report

b. Presentations: the PI and a PTSD clinical investigator colleague and consultant on this project, Dr. Murray Raskind, together presented a joint seminar at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC) and the VAPSHCS division of Research and Development in affiliation with the Seattle Institute of Biomedical and Clinical Research (SIBCR), entitled "Translation goes both ways; prazosin treatment from humans to rats and back".

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

8. REPORTABLE OUTCOMES: Our initial finding, that acoustic startle in alcohol-na'lve rats is highly predictive of subsequent voluntary IAA alcohol drinking and preference, extends and complements the results of one of our previous studies demonstrating that pre-stress acoustic startle predicts development of rat PTSD-like further increased acoustic startle and plasma corticosterone response following a traumatic stress. This suggests that increased acoustic startle and associated increased anxiety reflect underlying mechanisms that increase vulnerability to both PTSD and alcohol abuse. Together with our and others' previous results demonstrating that prazosin can decrease both voluntary alcohol intake and PTSD symptoms, these results strongly suggest that prazosin can be effective for both conditions and that an a1-adrenergic receptor-mediated mechanism is at least one component of the common underlying mechanism, and thus an especially appropriate target for intervention. The remaining studies further investigate these interactions, facilitating most effective translation of prazosin treatment to clinical utility. All necessary methodology and training has now been developed, trouble-shot and implemented, and these studies are progressing to successful completion. Our further development of the rat PTSD model, employing a single traumatic stress together with weekly brief contextual reminders of the stress will – together with the further characterization of PTSD-like responses in these studies – also provide a well-characterized experimental model for other labs investigating PTSD and alcohol abuse, alone or together. In addition, a) the findings, results and techniques of these studies are directly applicable to other investigations of the effects of stress or the evaluation of mechanisms contributing to voluntary alcohol and other drug abuse, b) the results of this investigation will facilitate

translating prazosin treatment to clinical implementation in the treatment of prazosin and alcohol abuse, alone or together, and c) our results will ultimately improve overall understanding and effective treatment of alcoholism and PTSD, two conditions with profound negative social and economic impact.

9. OTHER ACHIEVEMENTS: A great deal of basic and clinical PTSD and related traumatic brain injury (TBI) research is based at the VA Puget Sound Health Care System (VAPSHCS) Mental Illness Research, Education and Clinical Center (MIRECC), providing ample ongoing collaborative opportunities. The PI works closely with a PTSD clinical investigator colleague, Dr. Murray Raskind, as well as alcohol clinical investigators (Ors. Andrew Saxon and Tracy Simpson) and a TBI clinical investigator, Dr. Elaine Peskind, within the VAPSHCS and MIRECC to facilitate translation of basic science findings in the current investigation to clinical testing and future clinical implementation as discussed in the preceding section (8).

10. REFERENCES

Rasmussen DD, Kincaid CC. Acoustic startle in alcohol-na"ive male rats predicts subsequent voluntary alcohol intake and alcohol preference. Alcohol Alcohol. 50: 56-61, 2015 (doi: 10.1093/alcalc/agu065)

11. APPENDICES: One published paper, listed above in section 6, is appended.

Alcohol and Alcoholism Vol. 50, No. 1, pp. 56-61, 2015 doi: 10.1093/alcalc/agu065
Advance Access Publication 10 October 2014

Acoustic Startle in Alcohol-Naive Male Rats Predicts Subsequent Voluntary Alcohol Intake and Alcohol Preference

Dennis D. Rasmussen* and Carrie L. Kincaid

VISN 20 Mental Illness Research Education and Clinical Center, VA Puget Sound Health Care System and University of Washington, Seattle, WA 98195, USA
*Corresponding author: VA Medical Center, 116-MIRECC, 1660 S Columbian Way, Seattle, WA 98108, USA. Tel.: +1-206-277-3370; Fax: +1-206-768-5456; E-mail: drasmuss@u.washington.edu

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Abstract - Aims: Acoustic startle response in rats is used to model sensorimotor reactivity. The aim of the study was to determine whether acoustic startle response in alcohol-naive rats predicts subsequent increased voluntary alcohol drinking or alcohol preference. Methods: Startle responses to 90, 95 and 100 decibel (dB) white noise stimuli presented in counterbalanced semi-randomized order were tested in alcohol-naive young adult male Wistar rats before voluntary alcohol intake was established with an intermittent alcohol access (IAA) model. Results: Startle amplitude in response to 95 or 100 dB stimuli was positively correlated with subsequent alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split) pre-IAA startle amplitude in response to 95 or 100 dB stimuli developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA, relative to rats with low pre-IAA startle amplitude. Conclusion: Startle response to moderate acoustic stimuli can be a predictive index of vulnerability to developing increased alcohol drinking.

INTRODUCTION

Begleiter and Porjesz (1999) proposed that sensorimotor hyper-reactivity is a key feature of the simplest model of the neuronal milieu underlying a predisposition to alcoholism. This hypothesis is consistent with evidence that sensorimotor hyper-reactivity expressed as enhanced acoustic startle response is characteristic of abstinent alcoholics (Krystal *et al.*, 1997) and is associated with family history of alcoholism (Pfefferbaum *et al.*, 1991; Grillon *et al.*, 1997). Rats selectively bred for alcohol preference and high voluntary alcohol drinking (McKinzie *et al.*, 2000; Chester *et al.*, 2004; Acewicz *et al.*, 2012), and post-dependent rats experiencing either acute alcohol withdrawal or prolonged imposed alcohol abstinence (Rassnick *et al.*, 1992; Rasmussen *et al.*, 2005) also exhibit increased acoustic startle response.

Enhanced startle is associated with increased brain noradrenergic activation (Stevens *et al.*, 1994), and brain noradrenergic activation contributes to increased voluntary alcohol drinking (Walker *et al.*, 2008; Rasmussen *et al.*, 2009; Simpson *et al.*, 2009; Froehlich *et al.*, 2013; O'Neil *et al.*, 2013). Enhanced startle is also correlated with the increased anxiety (Morgan *et al.*, 1993; Davis *et al.*, 1997) that is common to many alcoholics (Cloninger, 1987; Kushner *et al.*, 2000) and that is a major risk factor for alcohol abuse (Koob and Le Moal, 1997). Furthermore, anxiety-related behavior in rats has been demonstrated to predict alcohol drinking under several schedules of alcohol access (Hayton *et al.*, 2012). We thus hypothesized that characterization of startle response may facilitate prospective identification of vulnerability to developing increased voluntary alcohol drinking and also may provide a basis for determining mechanisms mediating development of some alcohol use disorders. Accordingly, we investigated whether prospectively determined acoustic startle response in alcohol-naive rats was correlated with subsequent increased voluntary alcohol drinking or increased alcohol preference in an intermittent alcohol access (IAA) model. IAA, in which rats have access to 2-bottle choice (water vs 20% alcohol) home cage alcohol drinking for three 24-h sessions/week, separated by at least 24 h (e.g. Monday, Wednesday, Friday), has been reported to induce outbred Wistar rats to escalate alcohol intake over repetitive access sessions to achieve alcohol intake at individually variable high levels accompanied by high alcohol preference and blood alcohol concentrations (BACs) comparable to those achieved by selectively bred alcohol-preferring (P) rats, and has been suggested to effectively model some human alcohol use disorders (Wise, 1973; Simms *et al.*, 2008).

MATERIALS AND METHODS

Animals

Twenty-three alcohol-naïve young adult male Wistar rats (Simonsen Labs, Gilroy, CA, USA) weighing 285 ± 3 g were housed 2/cage in plastic shoebox cages with controlled temperature (21 ± 1°C) and a 12 h/12 h light/dark cycle (lights off at 0900 h). Standard rodent chow (Laboratory Rodent Diet #7001, Harlan Teklad, Madison, WI, USA) and water were available *ad libitum* throughout the study. All experimental procedures were approved by the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Acoustic startle testing system

Acoustic startle was tested with an SR-LAB Acoustic Startle System (SDI, San Diego, CA, USA) using a slight modification of methods we previously reported (Rasmussen *et al.*, 2008). Each SR-LAB test chamber includes a ventilated sound-attenuated cabinet containing a clear plastic cylindrical rat enclosure mounted on a piezoelectric accelerometer that detects muscle twitch in response to a brief pulse of white (mixed frequency) noise produced by a tweeter inside the cabinet. The force exerted on the accelerometer is digitized and expressed in millivolt (mV) units. Each startle response signal generated by the accelerometer was recorded as 65 consecutive 1 ms recordings, starting at the onset of each 40-ms startle tone. Results were analyzed as maximum peak

amplitude. Startle stimulus and background white noise levels were calibrated with a Radio Shack Digital Sound Level Meter (33-2055; RadioShack Corp., Fort Worth, TX, USA) placed in the center of the cylindrical rat enclosure. Each of 8 SR-LAB test chambers was calibrated before each use to provide 450 mV response to a consistent test stimulus provided by an SDI Standardization Unit (SDI, San Diego, CA, USA). Each SR-LAB test chamber and rat enclosure was cleaned with 0.5% Liquinox (Alconox, Inc., New York, NY, USA) before each use.

Pre-test acclimation

After acclimation to the animal colony room and the reversed light/dark cycle for 3 weeks, rats were transferred to a dark testing room adjoining the colony room for 90 min at 1.5-3 h after lights-off on each of 5 days prior to testing, with ambient 60 decibel (dB) white noise produced by a White Noise Generator (SDI, San Diego, CA, USA). On the first 4 days, each rat was acclimated to a dark SR-LAB test chamber for 5 min with 60 dB white background noise (produced by the tweeter inside the chamber) before being returned to the colony room, housed with the same cage mate. On the fifth day, each of the rats was likewise transferred to the dark testing room with ambient 60 dB white noise, placed in the dark SR-LAB test chamber for 5 min with 60 dB white background noise, and then exposed to 10 presentations of 40 ms 95 dB white noise pulses presented at 30 s intervals before being returned to the colony room. The goal of this initial exposure to acoustic pulses was to minimize effects of novelty stress in the subsequent acoustic startle testing with similar white noise pulses. Immediately following completion of the acclimation process, each rat was returned to the same colony room but individually housed in a plastic shoebox cage. All procedures during the acclimation and subsequent acoustic startle testing and IAA were conducted under dim red illumination.

Acoustic startle testing

Startle testing was conducted 7-10 days after completion of the pre-test acclimation. On the test day, rats were again transferred to the dark testing room with 60 dB background white noise. After 90 min, each rat was placed in a dark SR-LAB testing chamber with 60 dB background white noise for 5 min before quantitation of startle responses to 10 presentations each of 40 ms 90, 95 or 100 dB white noise pulses at 30 s intervals, with one pulse of each intensity (i.e. 90, 95 or 100 dB) in counterbalanced order within each of 10 sequential sets of three pulses (Table 1), with 60 dB background white noise between pulses. Thus, there were a total of 30 startle tests at 30 s i11tervals, with l0 tests/each of responses to 90, 95 or 100 dB pulses distlibuted in counterbalanced order over a 15-min peliod.

Table 1. Order of acoustic stimuli presentations

Sequential sets of 3 acoustic stimuli	Counterbalanced order of stimuli within sets (dB)		
J	90	95	JOO
2	95	JOO	90
3	JOO	90	95
4	90	95	JOO
5	95	JOO	90
6	JOO	90	95
7	90	95	JOO
8	95	100	90
9	JOO	90	95
JO	90	95	100

There were 30 s intervals between stimuli within each set as well as between each sequential set.

Alcohol drinking

Three weeks after startle testing, 2-bottle choice access to 20% (v/v) alcohol vs water was provided for 24 h/day, 3 days/week (M, W, F)-i.e. an IAA model (Wise, 1973; Simms *et al.*, 2008). The alcohol solution was prepared by diluting 95% alcohol (ethanol; Decon Labs, King of Prussia, PA, USA) with deionized water to make a 20% (v/v) solution. Alcohol (20%) and water were presented in ball-bearing sipper tubes, with positions of the tubes alternated in sequential alcohol access periods to control for potential side preferences. On days when alcohol was not provided, the rats had access to water only. On days when alcohol and water intakes were characterized for analysis, daily fluid intakes were determined by weighing each tube to the nearest 0.1 g. Alcohol and water tubes were also placed on two empty cages to determine loss due to spillage/leakage and evaporation; average losses in these two cages on each day were subtracted from intakes for that day. Net daily alcohol intake was converted to g alcohol/kg body weight. After 36 alcohol access days (i.e. 12 weeks, when stable alcohol intake was achieved) alcohol intake and alcohol preference (ml of alcohol intake/[ml of alcohol intake + ml of water intake]) were determined over the next 3 alcohol access days to characterize the relationships of each rat's alcohol intake and alcohol preference relative to its pre-IAA acoustic startle responses. One rat did not establish significant daily alcohol drinking (alcohol intake was <1 g/kg/day on all days evaluated) and was excluded from further analyses.

l)ata analyses

Startle amplitude in response to presentations of 90, 95 or 100 dB acoustic pulse intensities in counterbalanced order within each of 10 sequential sets of 3 pulse presentations were initially evaluated by two-way (set X pulse intensity) repeated measures analysis of variance (ANOVA) with repeated measures on sets (l-10) and pulse intensities (90, 95 or 100 dB). There was a significant effect of intensity, $F(2, 42) = 63.7$, $P < 0.001$, but no significant effect of set and no significant intensity x set interaction. Since startle amplitude in response to each of the acoustic stimulus intensities was independent of presentation time (set) within the 15 min test period, the average of all 10 responses to each stimulus intensity was used in subsequent analyses of the relationships between pre-IAA acoustic startle response vs IAA alcohol intake or alcohol preference. Similarly, IAA alcohol intake or alcohol preference on the 3 alcohol access days in IAA week 13 was analyzed by one-way ANOVA with repeated measures on day; there were no significant effects of day on either alcohol intake or alcohol preference, so 3-day average alcohol intake or 3-day average alcohol preference was likewise used in subsequent analyses of relationships between IAA week 13 alcohol intake or alcohol preference vs pre-IAA acoustic startle amplitude.

Pre-IAA startle amplitude in response to presentations of either 90, 95 or 100 dB stimuli was each compared with subsequent alcohol intake or alcohol preference by Pearson Product Moment Correlation Analysis. The pre-IAA startle response to

90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol intake was further compared by two-way (high vs low alcohol intake X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The pre-IAA startle amplitude in response to 90, 95 or 100 dB stimuli in rats grouped on the basis of high vs low (median split, $n = 11$/group) IAA week 13 alcohol preference was likewise compared by two-way (high vs low alcohol preference X stimulus intensity) ANOVA with repeated measures on stimulus intensity (90, 95, 100 dB). The IAA week 13 alcohol intake or alcohol preference of rats grouped on the basis of high vs low (median split, $n = 11$/group) pre-IAA startle amplitude in response to either 90, 95 or 100 dB stimuli was each analyzed by Student t-test (median splits of startle responses to each of the three stimulus intensities did not in each case identify the same animals to be included in the high vs low startle response groups, so two-way ANOVA testing could not be performed); Bonferroni c01Tections were not applied to individual t-tests.

All analyses were conducted using Sigmaplot Version 11 software (Systat Software, Inc., Chicago, IL, USA) with significance accepted at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

!AA alcohol intake and alcohol preference

Initial (i.e. IAA week l) average (M, W, F) alcohol intake was 0.95 ± 0.16 g/kg/24 h and alcohol preference was 0.07 ± 0.01. By IAA week 13, alcohol intake had increased to 3.80 ± 0.32 g/kg/24 h ($P < 0.001$) and alcohol preference had increased to 0.40 ± 0.03 ($P < 0.001$).

!AA alcohol intake relative to pre-IAA startle response

IAA week 1 alcohol intake was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.18$), 95 ($P = 0.11$) or 100 ($P = 0.15$) dB stimuli. IAA week 13 alcohol intake relative to pre-IAA startle amplitude elicited in response to presentations of either 90, 95 or l00 dB stimuli is presented in the upper, middle or lower row, respectively, of Fig. l.

Pre-IAA startle amplitude in response to 90 dB stimuli was modest and inconsistent; startle amplitude was not significantly c01Telated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, upper row, left). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol intake revealed no alcohol intake-dependent significant difference in pre-IAA startle in response to 90 dB stimuli (Fig. l, upper row, center; in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) x stimulus intensity (90, 95,l00 dB)] interaction, $F[2, 40] = 5.1$, $P \leq 0.01$, but pre-IAA startle amplitude in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol intake (Fig. 1, upper row, right).

Pre-IAA startle amplitude in response to 95 dB stimuli was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, middle row, left; $P < 0.01$, $r = 0.62$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol intake in IAA week 13 (Fig. 1, middle row, center; $P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle amplitude in response to 95 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 95 dB stimuli (Fig. 1, middle row, right; $P \leq 0.001$).

Pre-IAA startle amplitude in response to 100 dB stimuli also was positively correlated with alcohol intake established by 3 subsequent months of IAA (Fig. 1, lower row, left; $P < 0.01$, $r = 0.55$). Rats with high (median split) alcohol intake in IAA week 13 had previously exhibited greater pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, center; $P < 0.001$). Consistent with this results, rats with high (median split) pre-IAA sta1le response to 100 dB stimuli subsequently developed increased alcohol intake in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli (Fig. 1, lower row, right; $P < 0.01$).

!AA alcohol preference relative to pre-IAA startle response

IAA week 1 alcohol preference was not significantly correlated with pre-IAA startle amplitude elicited in response to 90 ($P = 0.19$), 95 ($P = 0.14$) or 100 ($P = 0.20$) dB stimuli.

IAA week 13 alcohol intake and alcohol preference were highly positively c01Telated, $r = 0.92$, $P < 0.001$. Further analyses of IAA week 13 alcohol preference relationships to pre-IAA startle responses were conducted identically to those in the preceding analysis of alcohol intake relationships to pre-IAA startle responses. Consistent with the high positive correlation between alcohol intake and alcohol preference, the results of analyses of alcohol preference vs pre-IAA startle responses, as detailed below, were essentially identical to the results of the preceding analyses of alcohol intake vs pre-IAA staitle responses. Pre-IAA startle amplitude in response to 90 dB stimuli was positively co!Telated with alcohol preference established by 3 subsequent months of IAA ($P < 0.05$, r = 0.47). Grouping the rats on the basis of high vs low (median split) IAA week 13 alcohol preference revealed no alcohol preference-dependent significant difference in pre-IAA staitle response to 90 dB stimuli (in the two-way ANOVA with repeated measures on stimulus intensity, there was a significant overall [alcohol intake (high, low) x stimulus intensity (90, 95, 100 dB)] interaction, $F[2, 40] = 6.41$, $P < 0.01$, but pre-IAA stai1le in response to the 90 dB stimulus was not significantly different between the high and low alcohol intake groups). Grouping on the basis of high vs low (median split) pre-IAA startle response to 90 dB stimuli likewise revealed no pre-IAA startle amplitude-dependent significant difference in IAA week 13 alcohol preference.

Pre-IAA startle amplitude in response to 95 dB pulses was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.57$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to 95 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.05$). Consistent with this result, rats with high (median split) pre-IAA startle response to 95 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA low startle response to 95 dB stimuli ($P < 0.01$).

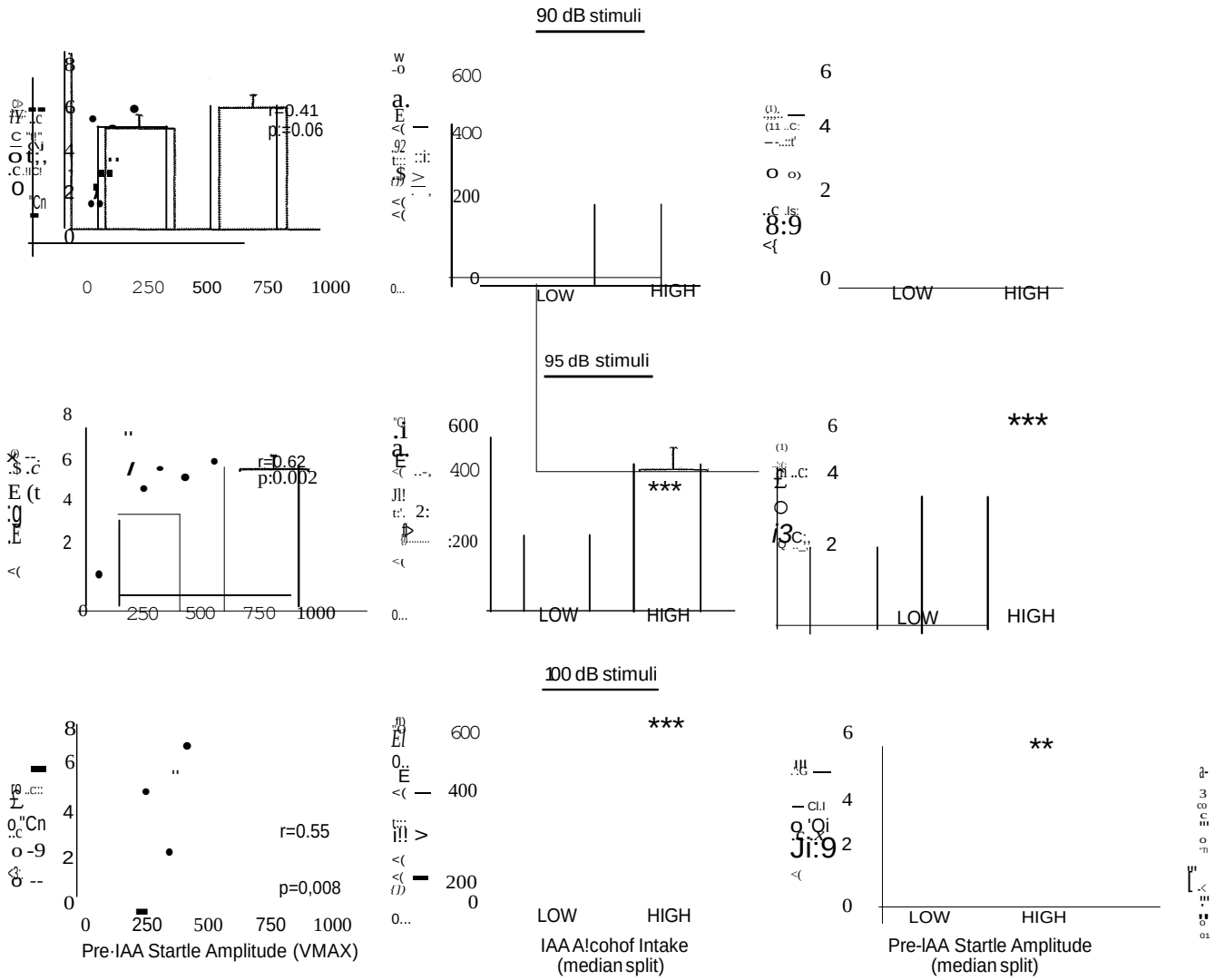

Fig. I. Pre-IAA acoustic startle response vs alcohol intake following 3 months of IAA. Rows: The upper, middle and lower rows present analyses of pre-IAA responses to 90, 95 or 100 dB acoustic startle stimuli, respectively. Columns: The left panel in each row presents the cotTelation between pre-IAA acoustic startle amplitude vs IAA week 13 alcohol intake for all 22 rats. The center panel in each row presents the pre-IAA acoustic startle amplitude of rats grouped on the basis of low vs high (median split, $n = 11$ rats/group) alcohol intake in IAA week 13. The right panel in each row presents the IAA week 13 alcohol intake of rats grouped on the basis oflow vs high (median split, $n = 11$ rats/group) pre-IAA acoustic staitle amplitude. **P s 0.01 vs Low, ***P s 0.001 vs Low.

Pre-IAA startle response to 100 dB stimuli also was positively correlated with alcohol preference established by 3 subsequent months of IAA ($P < 0.01$, $r = 0.53$). Rats with high (median split) alcohol preference in IAA week 13 had previously exhibited greater pre-IAA startle response to I 00 dB stimuli, relative to rats with low alcohol preference in IAA week 13 ($P < 0.001$). Consistent with this result, rats with high (median split) pre-IAA startle response to 100 dB stimuli subsequently developed increased alcohol preference in IAA week 13, relative to rats with low pre-IAA startle response to 100 dB stimuli ($P < 0.01$).

!AA alcohol intake relative to pre-IAA startle response to thefirst presentation of each stimulus intensity

Some previous investigations of alcohol d1inking in rats have compared alcohol intake or preference relative to startle amplitude in response to only the first presentation of an acoustic stimulus. In the current study, IAA week 13 alcohol intake or alcohol preference was not significantly correlated with pre-IAA startle in response to the first presentation of either 90 or 100 dB stimuli. However, pre-IAA startle amplitude in response to the first 95 dB stimulus was correlated with IAA week 13 alcohol intake ($r = 0.66$; P 0.001) as well as alcohol preference ($r = 0.56$; $P < 0.01$).

DISCUSSION

In alcohol-naive young adult male Wistar rats, acoustic startle amplitude in response to 40 ms pulses of white noise at intensities of 95 or 100 dB was positively correlated with subsequent voluntary alcohol intake and alcohol preference following 3 months of IAA. Rats with high (median split)

alcohol intake or alcohol preference following the 3 months of IAA had previously exhibited greater pre-IAA startle response to 95 as well as 100 dB stimuli, relative to rats with low alcohol intake or low alcohol preference. Conversely, rats with high (median split) pre-IAA startle response to 95 or 100 dB stimuli subsequently developed increased alcohol intake as well as increased alcohol preference following 3 months of IAA.

Stimulus intensities in this investigation were based on the results of preliminary trials with young male Wistar rats in which 90 dB stimuli produced inconsistent small startle responses, 95 or 100 dB stimuli reliably produced relatively consistent sub-maximal startle, and a higher intensity stimulus (120 dB) produced maximal responses. The moderate 90, 95 and 100 dB stimuli were selected in order to avoid ceiling effects that could compromise ability to differentiate responses between animals, as suggested by a repo1t that human startle amplitudes elicited by 90 dB, but not 114 dB, stimuli were positively correlated with number of previous alcohol detoxifications (Krystal *et al.,* 1997). It previously has been reported that male Wistar rats exhibited an inve1ted U-shaped curvilinear relationship between the startle response to an initial 120 dB acoustic stimulus vs later alcohol intake, and that startle habituation appeared to have predictive value regarding alcohol intake (Sandbak *et al.,* 2000). In the current study, habituation to repeated stimulus exposures was not apparent, and there were significant positive linear correlations between pre-IAA acoustic startle responses to 95 or 100 dB stimuli vs alcohol intake and alcohol preference following IAA. The apparent disparities between the Sandbak *et al. (2000)* study and the current study may be due to the differing stimulus intensities as well as to the incorporation of an initial session with exposure to repetitive moderate (95 dB) stimuli in advance of the testing trial in the current study in order to minimize novelty of the stimulus (consistent with clinical studies, in which the subjects are aware that they will hear acoustic stimuli during the testing trial). In addition, stimuli of three different intensities were presented in semi-random counterbalanced order throughout the 15 min trial in the current study, rather than consistent repetition of a single stimulus. It is also notable that the current study used an IA.A model of alcohol drinking in which a relatively high concentration of alcohol (20%, v/v) was available on 3 intermittent days each week, considered to be a model for excessive alcohol drinking (Wise, 1973; Simms *et al.,* 2008).

Alcohol-naive rats from lines selectively bred to prefer alcohol exhibit increased acoustic startle relative to selectively bred alcohol non-preferring rats (McKinzie *et al.,* 2000; Chester *et al.,* 2004; Acewicz *et al.,* 2012). Sons of alcoholics likewise exhibited increased acoustic startle compared with sons of non-alcoholic parents (Grillon *et aL* 1997). The current results suggest that mechanisms contributing to acous-· tic startle response have a functional role in the vulnerability to increased voluntary alcohol drinking, and that acoustic startle characterization can provide an index of sensorimotor hyper-reactivity and associated mechanisms that contribute to this increased alcohol drinking. Although these mechanisms remain to be resolved, it has been demonstrated that brain noradrenergic activation increases acoustic staitle response (Stevens *et al.,* 1994) and also produces sensorimotor hyperreactivity and anxiety (Redmoond and Huang, 1979; Sullivan *et al.,* 1999) which are major risk factors for development of alcohol use disorders (Cloninger, 1987; Koob and Le Moal, 1997; Begleitcr and Porjesz, 1999; Kushner *et al.,* 2000). Conversely, suppression of noradrenergic signaling not only decreases acoustic startle responses (Gresack and Risbrough, 2011; Olson *et al.,* 2011) but also decreases alcohol drinking in rats and humans (Walker *et al.,* 2008; Rasmussen *et al.,* 2009; Simpson *et al.,* 2009; Froehlich *et al.,* 2013; O'Neil *et al.,* 2013) and blocks the expression of increased alcohol drinking in rats selectively bred for alcohol intake (Froehlich *et a/,,* 2013). The consistent association of changes in acoustic stai-tle, anxiety and increased alcohol drinking with changes in noradrenergic signaling suggests that noradrenergic activation may have a key role in mediating the correlation between acoustic startle amplitude and subsequent development of increased voluntary alcohol drinking.

The current results demonstrate that acoustic staitle amplitude in response to moderately supra-threshold startle stimulus intensities administered to alcohol-na'ive male Wistar rats is an effective predictive index for subsequent increased voluntary alcohol intake and alcohol preference in the IAA model. Acoustic startle response may be an especially useful index of the vulnerability to developing increased alcohol drinking because it is not dependent upon, and potentially confounded by, interactions with other behaviors. Importantly, acoustic startle is also well-characterized for use in humans (Krystal *et al.,* 1997; GriUon and Baas, 2003; Grillon *et al.,* 1998, 2005), providing translational utility.

These results may provide a useful model for investigating neurobiological mechanisms mediating initiation and development of excessive alcohol drinking, as well as provide the conceptual basis for a potential approach to prospectively identifying individuals at increased risk for future alcohol use disorders, thus allowing potential preventive intervention.

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